Novel real-time RT-PCR for the sensitive detection of multiple MAGE gene transcripts

The present invention relates to a highly sensitive real-time RT-PCR method for specifically detecting the expression of more than one MAGE gene. The present invention further relates to a diagnostic composition for carrying out such a real-time RT-PCR as well as to oligonucleotides suitable for the cDNA synthesis reaction prior to real-time PCR amplification of more than one marker from the MAGE gene family.

The MAGE gene family was originally described in melanoma patients when cytolytic lymphocytes specific for the MAGE-A1 gene product were identified (van der Bruggen, Traversari et al. 1991). This gene was later found to belong to a cluster of 12 human MAGE-A genes located in the q28 region of the X chromosome and more recently other members of the family characterized as subfamilies MAGE-B, -C and -D were described (Chomez, De Backer et al. 2001). The biological function of MAGE gene products is not yet completely understood, but it is assumed that the genes play an important role in tissue regeneration and differentiation (Old 2001).

Selected members of the MAGE gene family (Table 1) are frequently expressed in many tumors almost irrespective of the histological origin but are completely silent in normal adult tissue with the single exception of testicular germ cells (De Plaen, Arden et al. 1994). Several MAGE gene products have been identified as promising targets for tumor immunotherapy and have already been used in vaccination trials. The gene products of MAGE-A1, -A2, -A3, -A4, -A6, -A10 and -A12 are frequently found to induce a cytolytic T-cell response in tumor patients and are therefore the most promising candidates to serve as specific indicators for cancer.

Recently, the exceptionally restricted expression of MAGE-A genes was exploited to develop a highly sensitive and tumor-specific multimarker nested RT-PCR based on the independent conventional amplification of MAGE-A1, -2, -3/6, -4 and -12, respectively (WO 98/46788). This approach was successfully applied for the sensitive detection of rare disseminated tumor cells in blood and bone marrow of various tumor patients with many different types of cancer (Kufer 2002). Others have established sensitive

conventional MAGE-PCR methods by use of consensus oligonucleotides that coamplify cDNA of several different MAGE genes (Park, Kwon et al. 2002). Such a pan-MAGE-PCR may also detect rare disseminated tumor cells with high sensitivity. However, it does not provide information on the MAGE gene expression pattern in individual cancer patients, as obtained with a multimarker MAGE PCR specifically amplifying several different individual members of the MAGE family.

Over the past decade the PCR technology made substantial progress through the development of rapid thermocylers and the introduction of fluorescence monitoring of amplified products after each cycle, enabling the quantification of gene expression with "*rapid-cycle real-time PCR*" assays (e.g. LightCycler® System, ABI PRISM® Sequence Detection System). Sensitive quantification of gene expression thereby relies on the detection of increasing fluorescence during the exponential phase of PCR proportional to the amount of nucleic acids in the sample at the beginning of the reaction. Quantification is based on the threshold cycle (C_T-value), the first cycle with detectable fluorescence, and can be performed in absolute manner with external standards or in relative manner with a comparative normalizing reference gene serving as internal calibrator. The determination of a non-inducable reference gene is a critical issue in real-time PCR, since even marginal variations in gene expression will inevitably alter the relative quantification profile of the target gene. Usually genes like glyceraldehyd-3-phospat dehydrogenase (GAPDH), porphobilinogen desaminase (PBGD), beta2-microglobin or beta-actin are frequently used as internal calibrators in real-time PCR. In comparison to conventional endpoint PCR the real-time assays display even higher sensitivity and precision as well as a shorter turnaround time for rapid analysis of the results. In general the fluorescence can be detected sequence-specific by use of hybridization probes or TaqMan® probes or sequence-unspecific by use of the SYBR Green I dye.

Recently, single MAGE markers have been amplified by use of the real-time PCR technique: Scanlan and coworkers (Scanlan, Gordon et al. 2002) investigated MAGE-A3 gene expression in tumor tissue by designing a gene-specific TaqMan probe for measuring mRNA quantity using an ABI 7700 sequence detection system. The group of Yoshioka (Yoshioka, Fujiwara et al. 2002) developed a real-time PCR including again the amplification of MAGE-A3 mRNA to screen for tumor cells in resected lymph nodes of cancer patients. Until today, however, there is no real-time PCR for utilizing the gene

expression of multiple MAGE markers for quantification of minimal residual tumor disease in cancer patients who have undergone successful treatment of their primary tumor but are at risk of developing distant metastasis growing from the seed of early disseminated tumor cells; these patients, whose tumor load can be advantageously determined by the method of the present invention, urgently need an adjuvant tumor therapy to prevent the seed of metastasis from growing.

The investigation of only single markers is bound to result in a dramatic loss of sensitivity, because of the expression heterogeneity of malignant tumors in general and of single disseminated tumor cells in particular. For these reasons a multimarker real-time RT-PCR method for the highly sensitive detection of multiple tumor relevant markers selected from the MAGE family, like e.g. MAGE-A1, -A2, -A3, -A4, -A6, -A10 and -A12, was highly preferable.

Accordingly, the present invention relates to a highly sensitive real-time RT-PCR for the specific and reliable detection of mRNA transcribed by rare tumor cells from more than one MAGE gene. On the one hand, PCR-primers described in the prior art (Kufer 2002; Park, Kwon et al. 2002) for conventional highly sensitive pan- or multimarker-MAGE amplification methods are also applicable for highly sensitive real-time RT-PCRs. On the other hand, however, methods for reverse transcription of mRNA as successfully used for conventional highly sensitive pan- or multimarker-MAGE RT-PCRs - unexpected from the prior art - turned out to be insufficient for corresponding highly sensitive real-time RT-PCRs.

Since those RT-PCRs designed to detect rare tumor cells, which rely on the amplification of a single marker gene only, are particularly susceptible to expression loss or down-regulation connected with the genomic instability and phenotypic heterogeneity of tumor cells, reliable detection of at least two different MAGE gene transcripts by the real-time RT-PCR of the present invention was absolutely required. Therefore, it is essential to make sure that each single member of the MAGE family selected as a marker for the highly sensitive real-time RT-PCR is reliably converted from mRNA to cDNA by reverse transcription with reproducible efficiency. Only under this prerequisite the relative content in biological samples to be analyzed of the different MAGE mRNA species compared to each other can be determined or quantification of

the different MAGE transcripts carried out in comparison to an internal calibrator template like the PBGD mRNA.

As a solution to this technical problem it has been found in the present invention, that reverse transcription of the different MAGE transcripts and optionally of the calibrator mRNA must be carried out simultaneously in a single cDNA-synthesis reaction, using highly selected oligonucleotide primers and sophisticated reaction conditions for reverse transcription, which could not be anticipated from the prior art.

Accordingly the present invention relates to a highly sensitive real-time RT-PCR capable of specifically detecting the expression of more than one MAGE gene, wherein reverse transcription of the corresponding MAGE transcripts is carried out simultaneously in a single cDNA-synthesis reaction. Carrying out efficient and reliable reverse transcription of different transcripts in a single cDNA-sythesis reaction was no trivial task, because the methods for reverse transcription (RT) of two or more different MAGE transcripts, which according to the prior art led to the reliable conversion of each single species of MAGE-mRNA into sufficient cDNA detectable by highly sensitive conventional PCRs, suprisingly failed to do so in combination with a highly sensitive real-time MAGE-PCR. Neither unspecific reverse transcription using cDNA-priming with oligo-dT or random hexanucleotides nor specific cDNA-priming with an established combination of mono- and dual-specific oligonucleotides hybridizing to the different MAGE-transcripts, respectively, proved to be sufficient for obtaining specific amplification products at the desired high sensitivity level for each member of the MAGE family selected as marker for the subsequent real-time PCR (Example 2). Even the use of a "pan-MAGE-primer" for cDNA-synthesis as taught by the closest prior art (WO 98/46788) disclosing a highly sensitive conventional multimarker MAGE RT-PCR did not succeed under standard conditions in sufficient reverse transcription of mRNA from every single MAGE gene used as marker in the corresponding real-time multimarker MAGE RT-PCR. Thus, it is required, in accordance with the present invention, to identify by careful testing under many different reaction conditions one or more primers for reverse transcription (=RT-primer), each hybridizing to the mRNA of one or more different members of the MAGE gene family. It is essential that the testing of RT-primers for the highly sensitive real-time MAGE RT-PCR is carried out in the presence of the whole cocktail of RT-primers during cDNA-synthesis. This is required because of frequent interferences among different RT-primers, which are neither predictable from

cDNA-synthesis reactions with only one RT-primer nor from the teachings of the prior art.

A real-time MAGE PCR, i.e. PCR-amplification of reverse transcribed MAGE-cDNA, in accordance with the present invention, can be implemented either by using a sequence unspecific DNA-dye like SYBR Green I or by applying sequence-defined fluorescent probes for the detection of specific amplicons. For carrying out the latter method, the sequences of MAGE mRNA molecules have to be screened for unique marker-defining regions if the detection of individual MAGE parameters is desired or pan-MAGE specific areas if the detection of several MAGE markers is desired in a single reaction. This unique hybridization region on the sequence must be located in between two oligonucleotides used as primers for the PCR and should neither be self-complementary, monotonous, or repetitive nor complementary to the PCR primers. The application of TaqMan probes requires the design of a single double-labeled fluorescent probe, for the application of hybridization probes the design of two fluorescent oligonucleotides is needed that hybridize in close proximity (1 to 5 bases) to each other on the amplicon to enable the distance-dependent transfer of energy between the fluorophores (fluorescence resonance energy transfer). The reaction conditions have to be carefully evaluated and optimized, involving the adaptation of primer and probe concentrations, temperatures and duration of PCR-cycling etc.

In a preferred embodiment of the method of the invention the MAGE genes serving as markers in a highly sensitive real-time RT-PCR are selected from the functional genes of MAGE subfamilies A, B and/or C (Table 1). Except for the pseudogenes, expression of the members of these MAGE-subfamilies is highly restricted to tumor cells, while they are completely silent in normal adult tissue with the only exception of testicular germ cells. Thus expression of functional MAGE A, B and/or C genes as detected by the highly sensitive real-time RT-PCR of the invention in blood, bone marrow, lymph nodes or other secondary organs of a tumor patient is highly indicative of the systemic spread of cancer cells from the primary tumor. Because of its quantitative nature, the highly sensitive real-time RT-PCR of the invention is particularly useful for measuring the load of disseminated tumor cells in individual patients, thus estimating the risk of a metastatic relapse originating from the early tumor cell spread that took place prior to successful

treatment of the primary tumor. Thus, the method of the invention may help to decide more precisely on the requirement of an adjuvant tumor therapy than is possible with diagnostic methods of the prior art.

Besides blood and bone marrow many kinds of body fluids or tissues, like urine, stool or sputum, are easily accessible to search for malignant cells. The real-time MAGE RT-PCR of the present invention is therefore also applicable as highly sensitive screening tool in secondary tumor prevention and can achieve the early detection of neoplasia particularly in individuals who are highly at risk of developing cancer.

In a particularly preferred embodiment of the method of the present invention the MAGE genes serving as markers in a highly sensitive real-time RT-PCR comprise MAGE-A 1, 2, 3, 4, 6, 10 and/or 12. These genes are most frequently expressed in many different types of tumors of various histological origins. Moreover, all members of this selected group of MAGE genes encode target antigens for cytotoxic T cells. Thus, the highly sensitive real-time RT-PCR of the invention will advantageously provide quantitative MAGE gene expression profiles of individual cancer patients as a basis for the rational design of MAGE-based tumor vaccines. So far, only qualitative patterns of MAGE gene expression could be obtained by using prior art methods making the choice of MAGE gene products to be included in a tumor vaccine more difficult.

In another preferred embodiment of the method of the invention at least one primer for reverse transcription of MAGE mRNA is selected from the following groups of oligonucleotides:

(A)

primer	sequence (5' - 3')
MgRT1a	CCA GCA TTT CTG CCT TTG TGA
MgRT1b	CCA GCA TTT CTG CCT GTT TG
MgRT2	CAG CTC CTC CCA GAT TT
MgRT3a	ACC TGC CGG TAC TCC AGG
MgRT3b	ACC TGC CGG TAC TCC AGG TA
MgRT4	GCC CTT GGA CCC CAC AGG AA
MgRT5a	AGG ACT TTC ACA TAG CTG GTT TCA
MgRT5b	GGA CTT TCA CAT AGC TGG TTT C
MgRT6	TTT ATT CAG ATT TAA TTT C

(B)

primer	sequence (5' - 3')
Mg1_RT1	CAA GAG AGA TGA TGA CTC TC
Mg1_RT2	TTC CTC AGG CTT GCA GTG CA
Mg1_RT3	GAG AGG AGG AGG AGG TGG C
Mg1_RT4	GAT CTG TTG ACC CAG CAG TG
Mg1_RT5a	CAC TGG GTT GCC TCT GTC
Mg1_RT5c	CTG GGT TGC CTC TGT CGA G
Mg1_RT5d	GGG TTG CCT CTG TCG AGT G
Mg1_RT5e	GGC TGC TGG AAC CCT CAC
Mg1_RT6	GCT TGG CCC CTC CTC TTC AC
Mg1_RT7	GAA CAA GGA CTC CAG GAT AC

Primers for reverse transcription as depicted in group A, are perfectly matching with each of the mRNA-sequences of MAGE-A 1, 2, 3, 4, 6, 10 and 12. However, most surprisingly, despite the perfect match, none of these RT-primers alone leads to sensitive detection of MAGE-A 1 expression by real-time RT-PCR under standard conditions as provided e.g. by the manufacturer of the LightCycler System. Under these recommended conditions the weakness in detection of MAGE-A 1 expression can only be compensated, as surprisingly found in the present invention, by combining two primers of group A with each other (e.g. *MgRT3a + MgRT5a*) or a group A-primer with one of the group B-primers, which are monospecific for the cDNA-synthesis of MAGE-A 1 only (Example 2). Depending on which at least two different members of the MAGE-A group encoding target antigens for cytotoxic T cells (i.e. MAGE-A 1, 2, 3, 4, 6, 10 and 12) are to be detected by real-time RT-PCR, different single RT-primers or combinations of RT-primers of group A and/or B may be applicable. In any individual case, however, in accordance with the present invention, careful testing of candidate RT-primers is required to end up with an optimal choice allowing the expression of the selected MAGE genes to be detected by real-time RT-PCR with a high level of sensitivity. As pointed out above, testing of RT-primers for the highly sensitive real-time MAGE RT-PCR has to be carried out in the presence of the whole cocktail of RT-primers during cDNA-synthesis, in order to cope with the unpredictable interferences among different RT-primers.

According to the teaching of the prior art (WO 98/46788) the average expert may - without undue burden - identify specific primers for the cDNA-synthesis that efficiently

hybridize to the mRNA of MAGE-1, -2, -3/6, -4 and -12. However, the whole series of "pan-MAGE primers" as depicted in group A failed to result in cDNA-synthesis, which would have allowed the highly sensitive detection of each single marker mRNA by real-time PCR as carried out according to the recommended protocol provided by the manufacturer of the LightCycler System (Roche). In every tested case the highly sensitive detection by real-time PCR of at least one mRNA-species from the group consisting of MAGE-1, -2, -3/6, -4 and -12 failed despite perfect hybridization of every pan-MAGE cDNA-primer to each of the corresponding transcripts (example 2). This clearly differs from the teaching of the above mentioned prior art document referring to a conventional multimarker MAGE RT-PCR.

In a further particularly preferred embodiment of the method of the present invention, in addition to the reverse transcription of MAGE transcripts, reverse transcription of a calibrator mRNA is simultaneously carried out in the same single cDNA-synthesis reaction followed by PCR-amplification of MAGE- and calibrator cDNAs. For making the quantitative results obtained by analysis of different blood-, bone marrow- or other tissue samples from one or more cancer patients using the real-time MAGE RT-PCR comparable with each other, a normalizing reference gene, in accordance with the present invention, is preferably included in the assay. In order to be capable of serving as internal calibrator the normalizing reference marker most preferably is an essentially non-inducible gene. It is further preferred that the expression level of the reference gene is comparable to the target gene(s) and constant in essentially all cells of the sample. Furthermore, in accordance with the present invention, it is critical that a specific cDNA-primer for reverse transcription (RT) of the calibrator mRNA is used as integral member of the RT-primer cocktail comprising the MAGE-specific cDNA-primers to guarantee equal assay conditions for both the different MAGE transcripts to be analysed and the reference marker.

In another preferred embodiment of the method of the present invention the normalizing reference gene serving as internal calibrator is porphobilinogen desaminase (PBGD), glyceraldehyd-3-phospat dehydrogenase (GAPDH), beta-2-microglobin or beta-actin.

In a further preferred embodiment of the method of the present invention the primer for reverse transcription of PBGD mRNA is selected from the following group of oligonucleotides:

primer	sequence (5' - 3')
PBGD_RT2	CAT ACA TGC ATT CCT CAG GGT
PBGD_RT3	GAA CTT TCT CTG CAG CTG GGC
PBGD_RT4	TGG CAG GGT TTC TAG GGT CT
PBGD_RT10a	GGT TTC CCC GAA TAC TCC TG
PBGD_RT10d	TTG CTA GGA TGA TGG CAC TG
PBGD_RT12b	CCA AGA TGT CCT GGT CCT TG
PBGD_RT12c	CAG CAC ACC CAC CAG ATC
PBGD_RT12d	AGA GTC TCG GGA TCG TGC
PBGD_RT12e	AGT CTC GGG ATC GTG CAG
PBGD_RT12f	TCT CGG GAT CGT GCA GCA
PBGD_RT12g	ATG CAG CGA AGC AGA GTC T
PBGD_RT12h	CCT TTC AGC GAT GCA GCG
PBGD_RT13a	GTA TGC ACG GCT ACT GGC
PBGD_RT14a	GCT ATC TGA GCC GTC TAG AC
PBGD_RT15a	AAT GTT ACG AGC AGT GAT GC
PBGD_RT15b	TGG GGC CCT CGT GGA ATG
PBGD_RT15e	CAG TTA ATG GGC ATC GTT AAG
PBGD_RT15f	ATC TGT GCC CCA CAA ACC AG
PBGD_RT15g	GGC CCG GGA TGT AGG CAC
PBGD_RT15h	GGT AAT CAC TCC CCA GAT AG
PBGD_RT15i	CTC CCG GGG TAA TCA CTC
PBGD_RT15j	CAG TCT CCC GGG GTA ATC
PBGD_RT15k	TGA GGA GGC AAG GCA GTC
PBGD_RT15l	GGA TTG GTT ACA TTC AAA GGC

For each individual real-time MAGE RT-PCR, however, in accordance with the present invention, careful testing of candidate RT-primers specific for PBGD or the mRNA of another reference gene together with the candidate MAGE RT-primer(s) is required to end up with an optimal choice allowing the expression of the selected MAGE genes to be measured by real-time RT-PCR in comparison with reliable expression signals from the reference gene at a high level of sensitivity. Also in this case, testing of RT-primers for the highly sensitive real-time MAGE RT-PCR including the RT-primer for reverse transcription of the internal calibrator mRNA has to be carried out in the presence of the

whole cocktail of RT-primers during cDNA-synthesis, in order to cope with the unpredictable interferences among different RT-primers.

In another embodiment of the method of the present invention the PCR-primers for amplification of PBGD-cDNA comprise oligonucleotides selected from the following groups:

PBGD sense primer	**sequence (5' - 3')**
hu_PBGD_se	AGA GTG ATT CGC GTG GGT ACC
PBGD_8	GGC TGC AAC GGC GGA AGA AAA C
PBGD_ 8_F	TGC AAC GGC GGA AGA AAA C
PBGD_ATG-Eco	ATG TCT GGT AAC GGC AAT GC

PBGD antisense primer	**sequence (5' - 3')**
PBGD_3	TTG CAG ATG GCT CCG ATG GTG AA
PBGD_3.1_R	GGC TCC GAT GGT GAA GCC
PBGD_R	TTG GGT GAA AGA CAA CAG CAT C

In an even more preferred embodiment of the method of the present invention oligonucleotides *hu_PBGD_se* and *PBGD_3.1_R* or *hu_PBGD_se* and *PBGD_R* are used as primer pairs for PCR-amplification of PBGD-cDNA.

Actually, to introduce PBGD as internal calibrator for quantification of MAGE transcripts by a highly sensitive real-time RT-PCR according to the present invention 24 different PBGD-specific cDNA-primers were designed and tested in the presence of the MAGE-specific cDNA-primers for efficient reverse transcription of PBGD-mRNA in a PBGD-specific real-time RT-PCR. Those PBGD-specific cDNA-primers, which gave good results in the subsequent PBGD-amplification by real-time PCR were then tested in combination with the MAGE-specific cDNA-primers in a quantitative multimarker MAGE real-time RT-PCR. However, it was found that no combination of three cDNA-primers each consisting of a pan-MAGE- and a MAGE-A1 specific cDNA-primer plus a PBGD-specific cDNA-primer led to the highly sensitive amplification of every single marker from the group consisting of MAGE-A1, -2, -3/6, -4, -10 and -12 transcripts by real-time RT-PCR (example 3). In order to solve this problem it turned out, that the reduction of cDNA-primers from a triple to a double combination was inevitable. For this purpose we

had to invent an RT-protocol using very sophisticated non-standard conditions comprising unusual primer concentrations and the use of a highly selected polymerase to make a single pan-MAGE cDNA-primer work together with a PBGD cDNA-primer, without loosing the highly sensitive amplification of only a single marker in the subsequent real-time PCR (example 3). This eventually led to a final protocol for a highly sensitive quantitative multimarker MAGE real-time RT-PCR which by no means could have been anticipated from the prior art (example 5).

Accordingly, a highly preferred embodiment of the method of the present invention is related to the use of not more than two different oligonucleotides (including the RT-primer of an internal calibrator) as primers for reverse transcription in the cDNA-synthesis reaction of the real-time MAGE RT-PCR of the present invention.
In a most preferred embodiment of the method of the present invention oligonucleotides *MgRT3a* and/or *Mg1_RT5a* are used as primers for reverse transcription in the cDNA-synthesis reaction.

In a further most preferred embodiments of the method of the present invention oligonucleotides *MgRT3a* and *PBGD_RT15b* are used as primers for reverse transcription in the cDNA-synthesis reaction.

In another embodiment of the method of the present invention the MAGE- and/or the calibrator-PCR are nested or semi-nested PCRs. In order to achieve the desired high sensitivity for detection of mRNA transcribed by rare tumor cells from more than one MAGE gene, the real-time RT-PCR may be designed as nested or semi-nested PCR. For this purpose a first round of cDNA-amplification may be carried out with an appropriate pair of PCR-primers either by conventional or real-time PCR. Most preferably, this first round of PCR should not proceed to the plateau phase of amplification. Otherwise, quantification of the template content in the sample to be analyzed by the method of the present invention may become very difficult or even impossible. Moreover, it may be preferable to stop such a first round of PCR in the early or middle linear phase of amplification instead of proceeding to the late linear phase, in order to avoid interferences of an excess of preamplified PCR-products with the subsequent round of real-time PCR. Accordingly, the number of PCR-cycles and the

reaction conditions that are appropriate for such a preamplification step have to be carefully optimized, respectively. In particular these parameters should be adapted to the distribution of template amounts in the collection of samples to be analyzed, to make sure, on the one hand, that the level of high sensitivity of the method of the invention is sufficient to detect MAGE in those samples showing very weak expression and, on the other hand, that quantification of MAGE in other samples showing higher expression is still feasible.

In a particularly preferred embodiment of the method of the present invention PCR-primers are used comprising pairs of oligonucleotides specifically amplifying only a single member of the selected group of MAGE genes, respectively. Despite the high homology among different members of the MAGE gene family, making the design of such monospecific oligonucleotides more difficult, a highly sensitive real-time MAGE RT-PCR for detecting the individual expression of more than one MAGE gene is highly preferable. Only thus, a quantitative expression profile of individual MAGE genes of rare disseminated tumor cells in individual cancer patients can be obtained, which may be essential for the selection of those members of the MAGE family to be included in an optimal tumor vaccine. Moreover, the prognostic impact of the expression levels of single members of the MAGE gene family may vary with different types of cancer, which can be analyzed with the real-time RT-PCR of the present invention only when pairs of PCR-primers monospecific for the cDNA of individual MAGE genes are used that do not crossamplify other members of the MAGE family.

In another embodiment of the method of the present invention PCR-primers are used comprising pairs of oligonucleotides amplifying more than one member of the selected group of MAGE genes, respectively (= pan-MAGE PCR).
Following reverse transcription real-time PCR amplification of MAGE cDNA with such consensus primers, like those suggested by Park et al. (Park, Kwon et al. 2002) may be carried out, which make use of the high level of sequence homology among the different MAGE gene transcripts. Using this or similar approaches real-time RT-PCR may lead to MAGE-amplification products derived from the transcripts of MAGE-A 1, -2, -3, -4, -6 and/or 12 expressed by as few as five cancer cells (e.g. from the human colon cancer cell line HT-29) in 10ml of blood, while staying negative with blood from healthy donors.

For detection of the real-time PCR-amplification product(s) the sequence-independent SYBR green I method can be applied using the LightCycler System; alternatively, sequence-specific fluorescent probes, e.g. TaqMan or hybridization probes may be used. Furthermore, tissue samples (e.g. bronchoscopic biopsies) from cancer patients with different types of tumors (e.g. non-small cell lung (NSCL) cancer) may be analyzed accordingly. For this embodiment of the method of the invention it is of particular advantage that the particular way of cDNA-synthesis disclosed by the present invention makes sure that each single member of the MAGE family selected as a marker for the highly sensitive real-time RT-PCR is reliably converted from mRNA to cDNA by reverse transcription with reproducible efficiency, because due to coamplification of cDNA from different MAGE genes drop-outs of single markers at the stage of reverse transcription may easily remain unrecognized in the PCR e.g. by a positive signal derived from only one marker thus pretending successful detection of other presumably coamplified markers that indeed may have failed sufficient cDNA-synthesis although being expressed.

In another particularly preferred embodiment of the method of the present invention the PCR-primers for amplification of MAGE-cDNA comprise oligonucleotides selected from one of the following groups:

(C)

PCR-primer	sequence (5' - 3')
MAGE-A1	GTA GAG TTC GGC CGA AGG AAC
MAGE-A1	CAG GAG CTG GGC AAT GAA GAC
MAGE-A2	CAT TGA AGG AGA AGA TCT GCC T
MAGE-A2	GAG TAG AAG AGG AAG AAG CGG T
MAGE-A3/6	GAA GCC GGC CCA GGC TCG
MAGE-A3/6	GAT GAC TCT GGT CAG GGC AA
MAGE-A4	CAC CAA GGA GAA GAT CTG CCT
MAGE-A4	TCC TCA GTA GTA GGA GCC TGT
MAGE-A10	CTA CAG ACA CAG TGG GTC GC
MAGE-A10	GCT TGG TAT TAG AGG ATA GCA G

MAGE-A12	TCC GTG AGG AGG CAA GGT TC
MAGE-A12	ATC GGA TTG ACT CCA GAG AGT A

(D)

PCR-primer	sequence (5' - 3')
MAGE-A1	TAG AGT TCG GCC GAA GGA AC
MAGE-A1	CTG GGC AAT GAA GAC CCA CA
MAGE-A2	CAT TGA AGG AGA AGA TCT GCC T
MAGE-A2	CAG GCT TGC AGT GCT GAC TC
MAGE-A3/6	GGC TCG GTG AGG AGG CAA G
MAGE-A3/6	GAT GAC TCT GGT CAG GGC AA
MAGE-A4	CAC CAA GGA GAA GAT CTG CCT
MAGE-A4	CAG GCT TGC AGT GCT GAC TCT
MAGE-A10	ATC TGA CAA GAG TCC AGG TTC
MAGE-A10	CGC TGA CGC TTT GGA GCT C
MAGE-A12	TCC GTG AGG AGG CAA GGT TC
MAGE-A12	GAG CCT GCG CAC CCA CCA A

This embodiment of the invention is advantageous because it is capable of measuring the individual expression of all those members of the MAGE-A subfamily encoding target antigens recognized by cytotoxic T lymphocytes, which are thus relevant for tumor vaccination. In the particular case of MAGE-A3 and 6, which are amplified by the same pairs of PCR-primers depicted in group C and D, there is no loss of information relevant for vaccine design caused by the coamplification, because the proteins encoded by MAGE-A3 and 6 are almost identical due to a sequence homology of 99%.

In an even more preferred embodiment of the method of the present invention primers of group C are used for a first round and/or primers of group D for a second round of PCR-amplification.
This embodiment of the invention is advantagous for carrying out a highly sensitive nested or semi-nested real-time MAGE RT-PCR.

In another embodiment of the method of the present invention a single or double pair of PCR-primers is used amplifying all members of the selected group of MAGE genes, respectively. This embodiment relates to a highly sensitive real-time RT-PCR specifically detecting the expression of more than one MAGE gene, by a single pair of

pan-MAGE PCR-primers in case of a single-step PCR or a double pair of pan-MAGE PCR-primers in case of a nested or semi-nested PCR. Due to the high level of sequence homology among the different MAGE genes, sites of sequence identity between all members of a selected group of MAGE genes may be found by computer-based sequence analysis, where such pan-MAGE PCR-primers can hybridize.

As with every pair of PCR-primers, either monospecific for the cDNA of an individual MAGE gene or oligospecific for the cDNAs of some or all members of a certain group of MAGE genes (= pan-MAGE PCR-primer), primer positions have to be selected in a way to avoid amplification of genomic MAGE DNA. For example, amplification of genomic MAGE-sequences can be avoided by the use of primers localized in different exons or primers spanning different neighboring exons, thus restricting hybridization to cDNA only. Furthermore, the positions of the PCR-primers have to be chosen to fall within the sequence segment(s) of the MAGE transcript(s), which is (are) reverse transcribed by the actual RT-primer(s) used for cDNA-synthesis.

The present invention further relates to a diagnostic composition comprising one or more suitable cDNA-primers for simultaneous reverse transcription of more than one different MAGE gene transcripts and optionally an appropriate calibrator mRNA in a single cDNA-synthesis reaction. The diagnostic composition of the invention is particularly useful for carrying out a variety of highly sensitive real-time MAGE RT-PCRs, thus allowing the quantification of the tumor cell load in cancer patients suffering from systemic tumor cell spread, by measuring the content of more than one kind of MAGE mRNA in blood-, bone marrow-, lymph node or other tissue samples. Moreover, the diagnostic kit is particularly useful for determining quantitative MAGE gene expression profiles of rare disseminated tumor cells in individual cancer patients, thus allowing the rational design of a MAGE-based tumor vaccine. In accordance with the present invention it is particularly preferable that at least one cDNA-primer of the diagnostic composition is MgRT3a, Mg1_RT5a or PBGD_RT15b.

Finally the present invention also relates to an oligonucleotide selected from the following group of primers:

MgRT3a

Mg1_RT5a
PBGD_RT15b

In accordance with the present invention, it was found that these oligonucleotides are particularly useful for simultaneously priming the reverse transcription of mRNA from more than MAGE genes in a single cDNA-synthesis reaction. It has been further found, in accordance with the present invention, (1) that this "single-pot" cDNA-synthesis is essential to make sure that each single member of the MAGE family selected as a marker for the highly sensitive real-time RT-PCR of the invention is reliably converted from mRNA to cDNA by reverse transcription with reproducible efficiency and (2) that only under this prerequisite the relative content in biological samples to be analyzed of the different MAGE mRNA species compared to each other can be determined or quantification of the different MAGE transcripts carried out in comparison to an internal calibrator template like the PBGD mRNA.

Definitions

The term

"RT" or "cDNA synthesis"	is used in the current invention for the conversion of mRNA into complementary DNA (cDNA) by a reverse transcriptase enzyme in a reverse transcription reaction (RT).
"RT-PCR"	is used in the current invention for methods applying a polymerase chain reaction (PCR) after converison of mRNA into complementary DNA (cDNA) by a reverse transcription reaction (RT).
"conventional PCR"	is used in the current invention for non-fluorescent PCR methods operated on all kinds of traditional thermocyclers.
"nested PCR"	is used in the current invention for PCR methods comprising two amplification steps with different sets of primers for the first and second round of amplification.

"semi-nested PCR" is used in the current invention for PCR methods comprising two amplification steps with one shared primer for the first and second round of amplification.

"real-time PCR" is used in the current invention for fluorescence-based PCR methods on photometric thermocyclers with the option for quantification of original template amounts. The method can include additional preamplification steps on a traditional thermocycler for a defined number of PCR-cycles.

"multimarker MAGE PCR" is used in the current invention for PCR assays that enable the separate amplification of cDNA of different individual MAGE genes.

"pan MAGE PCR" is used in the current invention for PCR assays that enable the amplification of cDNA of different MAGE genes by one or more pairs of consenus PCR-primers each capable of coamplifying at least two different MAGE gene transcripts.

"RT-primer" or

"cDNA synthesis primer" is used in the current invention for oligonucleotides designed to hybridize only to a defined target mRNA to yield specific cDNA molecules of these transcripts in a reverse transcription reaction.

"PCR primer" is used in the current invention for oligonucleotides designed to hybridize only to certain regions of target cDNA to yield amplicons of a specific length in a PCR reaction.

"high sensitivity" is used in the current invention for the capability of a PCR method to yield detectable MAGE specific amplificates from 5 or less tumor cells in 2 ml of whole blood. Additionally a crossing point below 30 PCR-cycles is required for real-time PCR-methods to fulfil the definition.

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For information on standard conditions known in the prior art the following references may be used

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Figure legends

Figure 1: Real-time amplification plot (A) and melting curve analysis (B) of MAGE-A1 transcripts in 2 ml of blood spiked with different numbers of Mz2-Mel cells as indicated using a standard LightCycler-DNA Master SYBR Green I protocol after oligo-dT primed cDNA synthesis. The arrows in B indicate the maximum of product dissemination over the indicated temperature range. The specific MAGE-A1 PCR product displays a melting peak at approximately 88.8°C. Unspecific products, e.g. primer dimers in this case, can be identified by their different dissociation curve. The gel electrophoresis (C) confirms specific amplification of the transcript and reliable detection of 1 tumor cell in 2 ml of whole blood.

Figure 2: Melting curve analysis after completion of a standard LightCycler-DNA Master SYBR Green I protocol for MAGE-A2 (A) and MAGE-A12 (B) after cDNA synthesis with oligo-dT priming. At least 10 tumor cells in 2 ml of blood are required for the generation of specific PCR products, samples with lower cell numbers do not result in specific signals with this protocol.

Figure 3: Real-time amplification plot (A) and melting curve analysis (B) of MAGE-A4 mRNA in 2ml of blood spiked with different numbers of LB-SAR cells as indicated. The first round of the nested PCR was performed with 15 cycles. Samples spiked with 5 and 10 LB-SAR cells yield the specific MAGE-A4 PCR product displaying a melting peak of approximately 87.6°C. Unspecific products obtained when spiking 1 cell or no cells can be distinguished by their different melting curves.

Figure 4: Real-time amplification plot (A) and melting curve analysis (B) of the same RNA sample applied in Figure 3 after 20 cycles of first PCR. The extension of preamplification leads to an improved sensitivity level with specific detection of MAGE-A4 PCR products when 1 LB-SAR tumor cell was diluted in 2 ml of blood. This positive achievement was associated with the increased formation of primer dimers in negative controls.

Figure 5:- Real-time amplification plot of MAGE transcripts after cDNA synthesis with a combination of antisense PCR primers for MAGE-1, -2, -3/6, -4 and -12. The application of 5 specific oligonucleotides in the reverse transcription reaction led to the formation of several unspecific products in the consecutive real-time PCR, e.g. primer dimers, associated with a deformation of the amplification curve.

Figure 6: Melting curve analysis of MAGE-A10 PCR products obtained with different primer-combinations for preamplification. Specific MAGE-A10 products are detectable only when a selection of two sense primers is used with a single antisense primer (*Mg10_anse5*). Approaches with the antisense primer *Mg10_anse4* for preamplification do not result in specific signals, although this oligonucleotide could be successfully applied as antisense primer in real-time PCR.

The examples illustrate the invention.

Example 1: Limitation of Oligo-dT primed cDNA synthesis for detecting MAGE transcripts expressed by rare tumor cells in blood with a real-time Multimarker MAGE-PCR

The first evaluation of a real-time MAGE PCR was performed in tumor cell dilution experiments. For this purpose we spiked 2 ml whole blood of healthy donors with different numbers of Mz2-Mel cells for amplification of MAGE-1, -2, -3/6 and -12 transcripts. To avoid degradation of the RNA each sample was immediately mixed with 10 ml denaturating nucleic acid extraction buffer [4 M guanidine isothiocyanate, 0.5% sarcosyl (N-laurylsarcosine sodium salt), 25 mM sodium citrate (pH=7.0), 0.7% 2-mercaptoethanol]. Total RNA was isolated according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi 1987) and was measured spectrophotometrically. cDNA was synthesized from 1 µg of total RNA by extension with 1.6 µg oligo-dT primer and 20 U avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany) at 25°C for 10 min., 42°C for 60 min. and 99°C for 5 min.

A first PCR round for preamplification was performed in 50 µl reactions containing 5 µl of cDNA, 5 µl of 10xPCR buffer (200 mM Tris, pH=8.0, 500 mM KCl), 1,5 µl of $MgCl_2$ (50µM), 4 µl of each dNTP (100µM) (Invitrogen, Groningen, Netherlands), 0.2 µM of each of the outer MAGE primers, and 1.25 units of Platinum Taq DNA-Polymerase (Invitrogen) and was run on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA) according to the following cycle profile: enzyme activation at 95°C for 3 min; denaturation at 95°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 15 cycles followed by terminal extension at 72°C for 7 min.

The quantification of MAGE gene expression was conducted using a LightCycler instrument and the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals, Mannheim, Germany). The inherent fluorescence of the SYBR Green I dye is enhanced 200-fold when it binds to the minor groove of double-stranded DNA. The increase in fluorescence is measured at the end of each cycle and indicates the amount of PCR products generated so far (F1, fluorescence channel 1 for SYBR

Green I). Because of the labeling of any double-stranded DNA nonspecific PCR products. e.g. primer dimers, will contribute to the signal. Therefore, the resulting PCR products in the SYBR Green I protocol are verified by means of a melting-curve analysis: since the dye only binds to double-stranded DNA, the fluorescent signal decreases as the melting point of the DNA duplex is reached. Following amplification the reaction mixture is subjected to an online melting curve analysis by increasing the temperature gradually (0.1°C/s).

The real-time PCR was carried out in 15 µl reaction mixture consisting of PCR grade water with 0.9 µl $MgCl_2$ (25mM), 1 µl of each inner MAGE primer (10pmol/µl), 1.5 µl of FastStart DNA Master SYBR Green I and 4 µl product of the first PCR reaction. Initial denaturation at 95°C for 5 min was followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 10 s and extension at 72°C for 20 s with a temperature slope of 20°C/s performed in LightCycler capillaries. All reactions were performed in duplicates and each run included a negative control without a template.

The results of the amplification of the MAGE-A1 gene product are shown in Figure 1: the amplification profile is depicted in Figure 1A with fluorescence on the Y axis and the number of the PCR cycles on the X axis. The cycle number at which the amplification curve crosses the baseline of the background signals is defined as *crossing point* and is used for quantification of the amount of template DNA in the sample. As expected more PCR cycles are required to amplify target cDNA in a sample containing less template cDNA. In this example it takes 22 cycles before fluorescence can be detected in the blood sample spiked with 100 tumor cells, the crossing points for the samples contaminated with 10, 5 and 1 cells follow consecutively. After 39 cycles there is also increase in fluorescence for the normal blood specimen, presumably caused by unspecific amplificates or primer-dimers.

Figure 1B shows the negative derivatives of the melting curve characteristics at the end of the PCR reaction. The peaks represent the *melting points* of the PCR product, i.e. the temperature at which 50% of the DNA PCR product melted. The melting curves for the MAGE-A1 PCR product display a characteristic melting point at 88.8°C. The unspecific amplificates in healthy blood, in this example primer dimers, show a different curve with a maximum at 87.5°C, enabling the discrimination of different PCR products and

verification of specificity. The gel electrophoresis in Figure 1C reconfirmes the specific amplification of the MAGE-A1 product for 1 to 100 tumor cell in 2 ml of blood.

The amplification of MAGE-A2 and -A12 did only yield specific signals when 10 tumor cells were added to 2 ml of blood. The increase of fluorescence with lower cell numbers was due to unspecific products (see Figures 2A and 2B).

Because of the lack of MAGE-A4 expression in Mz2-Mel cells we also spiked 2 ml whole blood of healthy donors with different numbers of LB-SAR cells that express MAGE-4 in a stable manner. The protocol was the same as described above but we used 2 µl of cDNA for PCR. The results of the amplification of the MAGE-4 gene product are shown in Figure 3. Any samples with less than 5 tumor cells per 2 ml did not result in specific detection of MAGE expression (Figure 3A). For 5 and 10 cells per 2 ml the real-time PCR yields the spefific MAGE-4 PCR product with a melting point of approximately 87.6°C, the melting curves for 0 and 1 cell reveal the amplification of unspecific products (Figure 3B).

To improve the sensitivity of the test we added 5 cycles to the protocol of the first PCR. The detection threshold was decreased thereby to 1 tumor cell / 2 ml of blood yielding specific MAGE-A4 products for all tumor cell dilutions (Figure 4A). However, the increase in PCR cycles was accompanied by primer-dimer formation causing a melting peak for the negative control without a template (Figure 4B). A further addition of PCR cylces for preamplification was impossible because of the reaching of a plateau phase with impracticability of consecutive quantification of gene expression.

This example shows the feasibility to detect the expression of single MAGE genes by real-time PCR, but it moreover clearly demonstrates the difficulty to ensure the amplification of <u>all</u> relevant MAGE mRNA species. Oligo-dT primed cDNA synthesis appears to prefer single mRNA molecules while omitting others. This property leads to the complete drop out of several parameters with a dramatic decrease in sensitivity to detect rare transcripts. The addition of more cycles in the first PCR round cannot completely compensate this loss because of the increasing appearance of unspecific products. PCR methods using consensus MAGE primers after reverse transcription with oligo-dT are especially susceptible for this considerable problem, because the putative "pan-MAGE" approach would only amplify some of the available MAGE transcripts. Altough the standard protocol for reverse transcription using oligo-dT primer is able to

generate successful amplification of MAGE-A1 transcipts it is not applicable for cDNA synthesis when screening for expression of the whole gene family.

Example 2: Superiority of highly selected MAGE-specific primers for cDNA-synthesis prior to amplification of MAGE by a real-time multimarker PCR

With the intention to establish a method for the sensitive detection of all MAGE mRNA subtypes and simultaneously decrease unspecific background we modified the AMV reverse transcription protocol by using a combination of the outer antisense PCR primers for MAGE-1, -2, -3/6, -4 and -12 in 2.5µM solution for specific cDNA synthesis. After preamplification with 20 cycles in a first PCR round the real-time PCR revealed a deformation of the amplification plot (Figure 5). The purification of the first PCR product led to the reconstitution of the regular amplification curve, presuming an overdosing of different oligonucleotides in the cDNA synthesis that causes interference of fluorescence in real-time PCR, although this approach could be shown to work in conventional PCR (Kufer 2002). The combination of several antisense primers for RT recation seems not to be an applicable approach for our purposes.

This accentuates the need for a specific shared oligonucleotide for reverse transcription of all MAGE-A mRNAs. We screened the MAGE-A sequences for universal segments by computer based sequence analysis and evaluated nine different oligonucleotides for their ability to act as sensitive pan-MAGE primer for the detection of MAGE-A transcripts in a 2 ml blood sample contaminated with 5 tumor cells (Mz2-Mel cells for MAGE-1, -2, -3/6 and -12; LB-SAR cells for MAGE-4) (Table 2). We used the standard *1st strand cDNA Synthesis Kit for RT-PCR (AMV)* supplied by Roche Molecular Biochemicals (Mannheim, Germany) in 20 µl with 2 µl of 10xReaction buffer, 5mM $MgCl_2$, 1mM of dNTP mixture, 50 units of RNAse inhibitor, 20 units of AMV reverse transcriptase and 1 µg of RNA according to the manufacturer's protocol and added specific oligonucleotide primers in 2.5µM concentration. The synthesis was perfomed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA) for 10 min at 25°C, 60 min at 42°C and 5 min at 99°C. Only one oligonucleotide turned out to be suitable for a reliable binding to all relevant MAGE-A mRNAs (Table 3), the other candidates as well as Oligo-dT, random hexamers or the combination of antisense PCR primers caused the drop out of at least one marker or displayed low sensitivity (C_T-

values >30). However, the transcription of the entire MAGE-A mRNAs by this single primer was accompanied by a late crossing-point for MAGE-A1 leading to poor sensitivity for this relevant marker.

In an attempt to improve the sensitivity for detection of the important MAGE-A1 marker we evaluated several additional reverse transcription primers specific for the synthesis of MAGE-A1 cDNA to be combined with the prior established pan-MAGE primer within the same protocol (Table 4). Again only one out of eleven combinations was able to amplify all relevant MAGE mRNA species, the other compositions showed drop outs of at least one marker (Table 5). Primer combinations failing to transcribe MAGE-A1 mRNA were excluded from further examination ("not determined" in the table). Meanwhile we included the MAGE-A10 marker to the protocol as decribed in example 4, nevertheless the combination of RT primers *MgRT3a* and *Mg1_RT5a* allowed the specific reverse transcription of all relevant MAGE-A mRNAs and the subsequent amplification in real-time PCR with much higher sensitivity than oligo-dT or random hexamers. This approach accomplishes the cDNA synthesis of the complete MAGE transcriptome and is therefore the prerequisite for sensitive PCR assays, irrespective of the used primers or PCR strategy.
Here we could document the detection of all relevant MAGE markers expressed in 5 tumor cells spiked into 2ml of whole blood. Furthermore the MAGE expression can be quantified using external standard curves or a direct comparison of single markers.

Example 3: Quantification of MAGE expression by use of an internal calibrator

The reliable quantification of gene expression implies the inclusion of an internal calibrator, e.g. a non-regulated housekeeping gene, to exclude variations in sample size or quality. Therefore we meant to quantify MAGE expression in relative comparison to the expression of a housekeeping gene, e.g. porphobilinogen-desaminase (PBGD). For this purpose we evaluated a broad variety of PBGD RT primers (Table 6) to integrate the reverse transcription of PBGD mRNA into the established cDNA synthesis protocol described in example 2. Primers that failed to yield specific amplification of PBGD transcripts were excluded from further evaluation. We tested 21 different PBGD specific oligonucleotides in combination with the prior established RT primers *MgRT3a* +

Mg1_RT5a, but only one combination using the primer *PBGD_RT10b* resulted in the successful generation of specific PCR products of all examined MAGE markers and PBGD (Table 7). However, the combination of three oligonucleotides in cDNA synthesis was linked to a markedly decreased sensitivity (crossing-points > 30 cycles for MAGE-2 and -12), thus appearing not to be an applicable approach when using real-time PCR subsequently. Therefore we were forced to evaluate further protocols with fewer than three RT primers.

Searching for more sensitive reverse transcriptase enzymes we tested the single pan-MAGE primer *MgRT3a* in two additional Kits for cDNA synthesis in a new experiment with 1 µg of total RNA from 2 ml of blood spiked with 5 tumor cells (Mz2-Mel cells for MAGE-1, -2, -3/6, -10, -12 and LB-SAR cells for MAGE-4). The *ThermoScript RT-PCR Kit* supplied by Invitrogen (Groningen, Netherlands) completely failed to create any amplifiable MAGE cDNA when we followed the standard protocol of the manufacture (Table 8), even though the Kit was designed for reverse transcription of difficult templates.

Additionally we applied an Omniscript RT Kit (Qiagen, Hilden, Germany) using the single pan-MAGE primer *MgRT3a* in 2.5µM solution [with 2µl of 10X Buffer RT, dNTPs 0.5mM each, 10 units of RNAse Inhibitor (Roche) and 4 units of Omniscript RT (Qiagen) for 60min at 37°C and 5 min. at 93°C] and could generate amplifyable cDNA of all relevant MAGE mRNAs and PBGD mRNA. The direct comparison of the Omniscript RT Kit to the prior used AMV Kit demonstrated a significantly higher sensitivity for the Omniscript RT reaction without drop outs of single parameters when analyzing identical aliquots of the same RNA preparation (Table 8).

This new protocol was tested on it's compatibility with various PBGD RT primers (Table 6) after the isolation of total RNA from 2 ml of blood spiked with 10 tumor cells. Combinations that failed to generate a positive PBGD signal were excluded from further evaluation (quoted as "not determined" in the table). Only one out of 32 oligonucleotides (*PBGD_RT15b*) did not disturb the reverse transcription of MAGE mRNAs while efficiently mediates the conversion of PBGD mRNA into cDNA (Table 9). The second best candidate (*PBGD_RT13a*) already failed to generate the same results when the sensitivity threshold was increased to 5 tumor cells / 2 ml blood. The crossing-point (C_T) differences clearly represent the varying levels of MAGE expression in the tumor cells with low amounts of MAGE-A10 and -A12 transcripts and high quantity of MAGE-A1

and -A4 mRNA in the utilized cell cultures as published in the literature (Serrano, Lethe et al. 1999). The expression level of each particular MAGE gene can be quoted as C_T^{MAGE} / C_T^{PBGD} and can therefore balance variations between individual samples. For the first time this protocol allows the reflection of the actual proportions of MAGE gene expression in striking contrast to the prior art.

Example 4: Detection of MAGE-A10 transcripts expressed by rare tumor cells in blood using real-time MAGE RT-PCR

In the first evaluation of the MAGE genes it was assumed that MAGE-A10 expression is only present at very low levels and therefore neglectable as marker for cancerous conditions. After the detection of MAGE-A10 specific cytolytic lymphocytes (Huang LQ, Brasseur F et al. 1999) the re-evaluation of expression profiles in tumor cells demonstrated weak but frequent transcription of the MAGE-A10 gene (Serrano, Lethe et al. 1999). Therefore it was our objective to include MAGE-A10 as another additional sensitive marker in the described Multimarker MAGE RT PCR.

A selection of different sense and antisense primers specific for MAGE-A10 cDNA were designed (Table 10) and tested for their potential to generate a MAGE-A10 PCR product. We isolated total RNA from 2 ml of whole blood contaminated with 100 Mz2-Mel cells and performed cDNA synthesis with the MAGE-RT primers MgRT3a and Mg1RT5a as described in example 2. The PCR was carried out in 50µl reactions with 10X PCR buffer (Invitrogen), dNTP mixture 0.2µM each (Invitrogen), 1.5µM $MgCl_2$, 1.25 units Platinum Taq DNA Polymerase (Invitrogen) and 2 µl of cDNA for 40cycles (initial enzyme activation at 95°C for 3 min, denaturation at 95°C for 30 sec, annealing at 60°C for 45 sec, elongation at 72°C for 60 sec and final extension at 72°C for 7 min). The PCR products were analyzed by electrophoresis in a 30% polyacrylamide gel and stained with ethidium bromide. Several primer combinations yielded specific MAGE-A10 PCR products (Table 11A). The most promising combinations were further evaluated for application in real-time PCR using a LightCycler FastStart SYBR Green I Kit. The reaction was performed in capillaries with a total volume of 15µl, 0.66µM PCR primer each and 2.5mM $MgCl_2$ concentration for 50 cycles (initial enzyme activation at 95°C for 5 min, denaturation at 95°C for 15 sec, annealing at 60°C for 10 sec and elongation at

72°C for 20 sec). The results demonstrated 6 successful primer combinations for the amplification of MAGE-A10 mRNA in the LightCycler system (Table 11B) that were used to construct a sensitive nested PCR for the detection of 5 Mz2-Mel cells in 2 ml of blood. Four different combinations were tested in a first PCR round with 20 cycles and subsequent real-time PCR analysis with the nested primer set *Mg10_se3* + *Mg10_anse2*. All experiments containing *Mg10_anse4* as antisense primer in the first PCR round failed to yield specific amplification (Figure 6), therefore primer sets including *Mg10_se5* as antisense primer in the first PCR are the only possible composition. The sense primers *Mg10_se1* or *Mg10_se3* can be successfully used for the first and second round of a nested or semi-nested PCR.

Example 5: Analysis of blood and bone marrow samples of cancer patients with a highly sensitive multimarker MAGE real-time RT-PCR

After careful optimization of the reaction conditions we tested blood and bone marrow samples from patients with localized prostate cancer for disseminated tumor cells with the multimarker MAGE real-time RT-PCR. 1 ml of bone marrow aspirate and 2 ml of blood were stabilized and prepared according to example 1. Total RNA was resuspended in 50 µl of DEPC-treated water and 10 µl were utilized in the subsequent cDNA synthesis using the Omniscript RT Kit (Qiagen, Hilden, Germany) in 20 µl with 2 µl of 10xBuffer RT, 2 µl of the supplied dNTP mix, 1 µl of MgRT3a (50pmol/µl) and PBGD_RT15b primer (50pmol/µl), 10 units of RNAse inhibitor (Roche) and 4 units of Omniscript RT enzyme. The reaction was executed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA) for 60 min at 37°C followed by denaturation at 93°C for 5 min.

2 µl of the cDNA were used for the first round of PCR in 20 µl reactions with 2 µl of 10xPCR Buffer, 0.6µl $MgCl_2$ (50µM), 1.6 µl dNTP mix and 0.2 µl Platinum Taq DNA polymerase (all by Invitrogen, Groningen, Netherlands) and 0.4 µl of each outer MAGE primer (10pmol/µl) according to the protocol described in example 1. For the real-time PCR we prepared 15 µl reactions in LightCycler capillaries with 1.5 µl of FastStart DNA Master SYBR Green I reagent, 2 µl of the first PCR product and different concentrations

of $MgCl_2$ and each inner MAGE primer: for MAGE-A1 we used 2.5mM $MgCl_2$ and 1 µM inner MAGE-A1 primers, for MAGE-A2 and -A10 3mM $MgCl_2$ and 1.2 µM inner MAGE-A2 or -A10 primers, for MAGE-A3/6 and -4 2.5mM $MgCl_2$ and 1.2 µM inner MAGE-A3/6 or -4 primers, and for MAGE-A12 3mM $MgCl_2$ and 0.8 µM inner MAGE-A12 primers. The reaction was run for 5 min at 95°C for initial activation of the enzyme, 10 sec at 95°C for denaturation, 5 sec at 60°C for annealing and 10 sec at 72°C for elongation for 40 cycles. After completion of the reaction the PCR products were subjected to a melting curve analysis spanning 65°C to 95°C with a ramping rate of 0.1°C/s and confirmed with electrophoresis on 30% acrylamide gels in ambiguous cases.

The amplification of PBGD mRNA was performed in a separate real-time PCR in 20 µl with 1µl of cDNA, 5mM $MgCl_2$, 0.5µM of sense primer (5'- AGA GTG ATT CGC GTG GGT ACC - 3'), 0.5µM of antisense primer (5'- TTG GGT GAA AGA CAA CAG CAT C - 3') and 2 µl of FastStart DNA Master SYBR Green I (Roche). The protocol was modified as follows: initial enzyme activation for 5 min at 95°C, denaturation for 15 sec at 95°C, annealing at 60°C for 10 sec and extension for 20 sec at 72°C. After completion of the PCR the products were subjected to a melting curve analysis as described before.

In total we were able to screen two groups of patients with prostate cancer after radical prostatectomy:

(a) 21 patients with attested biochemical relapse after radical prostatectomy defined as rising serum PSA level > 0.5ng/ml in the absence of any signs for local tumor growth. These patients bear a high risk for developing metastatic disease because of systemic spread of disseminated PSA producing tumor cells.

(b) 18 patients without biochemical relapse after radical prostatectomy defined as serum PSA level < 0.5ng/ml and presentation of a low risk profile for systemic tumor spread (i.e. Gleason score 6 and preoperative serum PSA level 20ng/ml and tumor stage pT_1 or pT_2, pN_0, R_0) and postoperative survival > 30 months at the time of sample collection. In these patients the development of metastatic disease should be an unlikely event.

In the high risk group we could identify 14 patients (= 66%, Table 12) with the expression of at least one MAGE gene in at least one sample (bilateral bone marrow aspirates or blood). The low risk group displayed MAGE expression in samples of 7

patients (= 38%, Table 13). Furthermore the total number of positive tests as well as the expression level was much higher in the high risk than in the low risk cohort. It must be emphasized that the low risk group does not represent a true "negative" or "control" group, since these patients also had malignant disease. Because of the generally long disease free survival time of patients with prostate cancer the detection of MAGE gene expression in the low risk group can be interpreted as true positive results with evidence for systemic tumor spread before clinical manifestation of metastatic disease at the present time. The quantity of MAGE expression can add further prognostic value, but the potency of these parameter has to be assessed after completion of the follow-up period of the cohort. The analysis of this cohort provides an impressive prove of principle for the sensitive detection and quantification of several individual MAGE markers by the real-time RT-PCR of the present invention and the exemplified use of this method for early diagnosis of minimal residual tumor disease.

Table 1: Members of the MAGE gene family showing restricted expression in malignant tumors and testicular germ cells only.

Gene Subfamily	Gene Name
MAGE-A	hMAGE-A1
	hMAGE-A2
	hMAGE-A3
	hMAGE-A4
	hMAGE-A5
	hMAGE-A6
	hMAGE-A8
	hMAGE-A9
	hMAGE-A10
	hMAGE-A11
	hMAGE-A12
MAGE-B	hMAGE-B1
	hMAGE-B2
	hMAGE-B3
	hMAGE-B4
	hMAGE-B5
	hMAGE-B6
	hMAGE-B10
	hMAGE-B16
	hMAGE-B17
MAGE-C	hMAGE-C1
	hMAGE-C2
	hMAGE-C3
	hMAGE-C4

Table 2: Oligonucleotides used as primers for specific pan-MAGE reverse transcription

primer	sequence (5' - 3')
MgRT1a	CCA GCA TTT CTG CCT TTG TGA
MgRT1b	CCA GCA TTT CTG CCT GTT TG
MgRT2	CAG CTC CTC CCA GAT TT
MgRT3a	ACC TGC CGG TAC TCC AGG
MgRT3b	ACC TGC CGG TAC TCC AGG TA
MgRT4	GCC CTT GGA CCC CAC AGG AA
MgRT5a	AGG ACT TTC ACA TAG CTG GTT TCA
MgRT5b	GGA CTT TCA CAT AGC TGG TTT C
MgRT6	TTT ATT CAG ATT TAA TTT C

Table 3: Evaluation of different primers33for cDNA synthesis: Crossing-Points for the detection of MAGE mRNA by a multimarker MAGE real-time RT-PCR. "n.d." = not determined, "-" = negative signal

RT-Primer	*MAGE-1*	*MAGE-2*	*MAGE-3/6*	*MAGE-4*	*MAGE-12*
random hexamer	33,3	n.d.	n.d.	n.d.	n.d.
Oligo-dT	30,1	-	27,0	30,2	-
3' primer combination	30,2	24,8	26,5	23,7	-
MgRT1a	28,8	-	-	31,4	-
MgRT1b	-	22,0	20,9	35,1	-
MgRT2	-	24,17	23,0	29,9	34,5
MgRT3a	30,2	22,7	19,8	21,7	22,1
MgRT3b	28,7	-	-	21,8	-
MgRT4	-	23,4	22,4	20,7	-
MgRT5a	27,6	-	20,6	22,4	-
MgRT5b	28,4	-	-	20,0	-
MgRT6	-	-	21,6	-	25,0

Table 4: Oligonucleotides used as34primers for MAGE-A1 specific reverse transcription

primer	sequence (5' - 3')
Mg1_ RT1	CAA GAG ACA TGA TGA CTC TC
Mg1_RT2	TTC CTC AGG CTT GCA GTG CA
Mg1_RT3	GAG AGG AGG AGG AGG TGG C
Mg1_RT4	GAT CTG TTG ACC CAG CAG TG
Mg1_RT5a	CAC TGG GTT GCC TCT GTC
Mg1_RT5c	CTG GGT TGC CTC TGT CGA G
Mg1_RT5d	GGG TTG CCT CTG TCG AGT G
Mg1_RT5e	GGC TGC TGG AAC CCT CAC
Mg1_RT6	GCT TGG CCC CTC CTC TTC AC
Mg1_RT7	GAA CAA GGA CTC CAG GAT AC

Table 5: Combination of pan-MAGE RT35primer MgRT3a with different MAGE-A1 specific RT primers for cDNA synthesis: Crossing-Points for the detection of MAGE mRNA by a multimarker MAGE real-time RT-PCR. "n.d." = not determined, "-" = negative signal

RT-Primer: MgRT3a +	*MAGE-1*	*MAGE-2*	*MAGE-3/6*	*MAGE-4*	*MAGE-10*	*MAGE-12*
Mg1 outer 3'	20,6	23,9	23,2	n.d.	-	-
Mg1 RT1	19,8	-	21,7	n.d.	n.d.	-
Mg1 RT2	-	21,5	19,5	n.d.	n.d.	-
Mg1 RT3	-	n.d.	n.d.	n.d.	n.d.	n.d.
Mg1 RT4	-	n.d.	n.d.	n.d.	n.d.	n.d.
Mg1 RT5a	20,7	22,1	12,1	20.9	23,5	23,5
Mg1 RT5c	20,3	28,7	29,3	n.d.	-	-
Mg1 RT5d	22,1	21,3	29,3	n.d.	-	-
Mg1 RT5e	22,2	25,0	33,7	n.d.	24,1	22,8
Mg1 RT6	-	n.d.	n.d.	n.d.	n.d.	n.d.
Mg1 RT7	22,0	26,2	20,9	n.d.	25,9	-

Table 6: Oligonucleotides used as36primers for specific reverse transcription of PBGD mRNA

primer	sequence (5' - 3')
PBGD_R	TTG GGT GAA AGA CAA CAG CAT C
PBGD_3	TTG CAG ATG GCT CCG ATG GTG AAG
PBGD_3.1 R	GGC TCC GAT GGT GAA GCC
PBGD_RT1	AAC TCC TGC TGC TCG TCC AG
PBGD_RT2	CAT ACA TGC ATT CCT CAG GGT
PBGD_RT3	GAA CTT TCT CTG CAG CTG GGC
PBGD_RT4	TGG CAG GGT TTC TAG GGT CT
PBGD_RT5	TTG TGC CAG CCC ATG CGC TG
PBGD_10a	GGT TTC CCC GAA TAC TCC TG
PBGD_10b	AGC TTC CGA AGC CGG GTG
PBGD_10d	TTG CTA GGA TGA TGG CAC TG
PBGD_12a	CTT GGC TCG CAC TTC CAC G
PBGD_12b	CCA AGA TGT CCT GGT CCT TG
PBGD_12c	CAG CAC ACC CAC CAG ATC
PBGD_12d	AGA GTC TCG GGA TCG TGC
PBGD_12e	AGT CTC GGG ATC GTG CAG
PBGD_12f	TCT CGG GAT CGT GCA GCA
PBGD_12g	ATG CAG CGA AGC AGA GTC T
PBGD_12h	CCT TTC AGC GAT GCA GCG
PBGD_13a	GTA TGC ACG GCT ACT GGC
PBGD_14a	GCT ATC TGA GCC GTC TAG AC
PBGD_14b	GCA GGG ACA TGG ATG GTA G
PBGD_15a	AAT GTT ACG AGC AGT GAT GC
PBGD_15b	TGG GGC CCT CGT GGA ATG
PBGD_15c	AGC CAA CTG GGG CCC TCG
PBGD_15d	TAA GCT GCC GTG CAA CAT CC
PBGD_15e	CAG TTA ATG GGC ATC GTT AAG
PBGD_15f	ATC TGT GCC CCA CAA ACC AG
PBGD_15g	GGC CCG GGA TGT AGG CAC
PBGD_15h	GGT AAT CAC TCC CCA GAT AG
PBGD_15i	CTC CCG GGG TAA TCA CTC
PBGD_15j	CAG TCT CCC GGG GTA ATC
PBGD_15k	TGA GGA GGC AAG GCA GTC
PBGD_15l	GGA TTG GTT ACA TTC AAA GGC

Table 7: Combination of primers *MgRT3a* and *Mg1_RT5a* with PBGD specific RT primers for cDNA synthesis: Crossing-Points for the detection of PBGD and MAGE mRNA by a multimarker MAGE real-time RT-PCR. "n.d." = not determined, "-" = negative signal

RT-Primer: MgRT3a + Mg1_RT5a +	*PBGD*	*MAGE-1*	*MAGE-2*	*MAGE-3/6*	*MAGE-4*	*MAGE-10*	*MAGE-12*
PBGD_R	-	-	24,5	24,9	20,9	n.d.	21,9
PBGD_3	-	20,3	21,8	20,1	n.d.	n.d.	-
PBGD_3.1_R	-	-	22,6	20,2	n.d.	n.d.	-
PBGD_RT1	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT2	14,3	22,3	21,8	-	n.d.	n.d.	28,7
PBGD_RT3	13,4	23,0	22,6	20,3	n.d.	n.d.	-
PBGD_RT4	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT5	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT10a	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT10b	13,0	23,2	31,6	21,3	n.d.	24,6	33,3
PBGD_RT10d	14,1	22,7	23,8	21,1	n.d.	-	30,0
PBGD_RT12a	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT12b	14,4	29,0	21,6	32,0	n.d.	-	-

Table 7, continued:

RT-Primer: MgRT3a + Mg1_RT5a +	*PBGD*	*MAGE-1*	*MAGE-2*	*MAGE-3/6*	*MAGE-4*	*MAGE-10*	*MAGE-12*
PBGD_RT12c	14,6	28,0	20,8	>36	n.d.	-	>36
PBGD_RT12d	15,2	26,0	22,3	>36	n.d.	-	>36
PBGD_RT12e	14,3	26,8	23,2	21,2	n.d.	-	>36
PBGD_RT12f	14,6	21,2	-	21,9	n.d.	-	-
PBGD_RT12g	14,9	21,8	29,4	23,3	n.d.	-	22,6
PBGD_RT12h	15,2	-	22,7	21,5	n.d.	-	-
PBGD_RT13a	14,0	-	23,5	-	n.d.	-	24,5
PBGD_RT15a	14,5	21,9	25,8	20,9	n.d.	-	31,3

Table 8: Evaluation of different reverse transcriptase enzymes: Crossing-Points for the detection of PBGD and MAGE mRNA by a multimarker MAGE real-time RT-PCR. "n.d." = not determined, "-" = negative signal

RT Enzyme:	*MAGE-1*	*MAGE-2*	*MAGE-3/6*	*MAGE-4*	*MAGE-10*	*MAGE-12*
Thermoscript (Invitrogen)	-	-	-	n.d.	-	-
AMV (Roche)	-	26,0	19,4	n.d.	-	22,3
Omniscript (Qiagen)	19,6	22,1	19,8	16,9	23,8	21,5

Table 9: Combination of pan-MAGE RT 42primer MgRT3a with different PBGD RT primers using the Omniscript RT protocol: Crossing-Points for the detection of PBGD and MAGE mRNA by a multimarker MAGE real-time RT-PCR. "n.d." = not determined, "-" = negative signal

RT Primer: MgRT3a +	*PBGD*	43 *MAGE-1*	*MAGE-2*	*MAGE-3/6*	*MAGE-4*	*MAGE-10*	*MAGE-12*
PBGD_R	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT1	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT2	22,7	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT3	18,9	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT4	19,8	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT5	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT10a	16,3	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT10b	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT10d	22,6	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT12a	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT12b	18,0	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT12c	21,6	21,0	-	32,2	n.d.	35,7	-
PBGD_RT12d	25,6	21,6	25,1	30,1	n.d.	-	-
PBGD_RT12e	18,6	22,2	23,0	25,8	n.d.	-	-
PBGD_RT12f	20,8	22,8	-	30,1	n.d.	-	-
PBGD_RT12g	22,9	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT12h	23,6	20,3	22,1	26,7	n.d.	-	-

Table 9: Combination of pan-MAGE RT 42primer MgRT3a with different PBGD RT primers using the Omniscript RT protocol: Crossing-Points for the detection of PBGD and MAGE mRNA by a multimarker MAGE real-time RT-PCR. "n.d." = not determined, "-" = negative signal (CONTINUED)

RT Primer: MgRT3a +	*PBGD*	43 *MAGE-1*	*MAGE-2*	*MAGE-3/6*	*MAGE-4*	*MAGE-10*	*MAGE-12*
PBGD_RT13a	21,4	21,1	23,7	20,1	n.d.	30,0	25,6
PBGD_RT14a	23,7	22,8	29,2	27,0	n.d.	-	>33
PBGD_RT14b	-	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT15a	23,8	19,7	25,9	22,0	n.d.	-	24,6
PBGD_RT15b	**20,2**	**20,3**	**22,2**	**28,9**	**23,1**	**25,5**	**21,7**
PBGD_RT15c	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PBGD_RT15d	-	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT15e	18,5	20,3	22,0	33,0	n.d.	-	23,9
PBGD_RT15f	19,9	19,8	27,0	18,0	n.d.	22,6	-
PBGD_RT15g	18,5	21,2	23,3	29,0	n.d.	-	-
PBGD_RT15h	22,7	-	n.d.	n.d.	n.d.	n.d.	-
PBGD_RT15i	22,0	-	26,2	19,8	n.d.	-	-
PBGD_RT15j	24,6	18,8	20,7	25,7	n.d.	-	-
PBGD_RT15k	22,7	18,7	20,0	26,5	n.d.	-	22,3
PBGD_RT15l	24,2	-	21,8	26,6	n.d.	23,2	19,6

Table 10: Oligonucleotides used as PCR primers for the amplification of MAGE-A10 cDNA

primer	sequence (5' - 3')
Mg10_se1	CTA CAG ACA CAG TGG GTC GC
Mg10_se2	GCA GGA TCT GAC AAG AGT CC
Mg10_se3	ATC TGA CAA GAG TCC AGG TCC
Mg10_anse1	TGG GAG TGT GGG CAG GAC T
Mg10_anse2	CGC TGA CGC TTT GGA GCT C
Mg10_anse3	ATC CTC CTC CAC AGC CAG G
Mg10_anse4	GGA GCT GGT GGA AGT GGA TG
Mg10_anse5	GCT TGG TAT TAG AGG ATA GCA G
Mg10_anse6	CAT CAG CAG AAA CCT CCT CTG
Mg10_anse7	AAT GGA AGG GAA GCA ACG ACC
Mg10_anse8	GGA GCC CTC ATC AGA TTG ATC

Table 11: Combination of MAGE-A1046specific sense and antisense PCR primer

Results of conventional RT-PCR with 40 cycles using total RNA isolated from 2ml of blood spiked with 100 Mz2/Mel cells.

primer combination	Mg10_se1 +	Mg10_se2 +	Mg10_se3 +
Mg10_anse1	-	-	+
Mg10_anse2	+	-	+++
Mg10_anse3	-	-	+
Mg10_anse4	++	+	++
Mg10_anse5	++	+	++
Mg10_anse6	+	+	+
Mg10_anse7	+	(+)	+
Mg10_anse8	-	-	+

"(+)" - "+++" = intensity of specific signal, "-" = no signal

Results of real-time PCR with 50 cycles using total RNA isolated from 2ml of blood spiked with 100 Mz2/Mel cells: Crossing-Points for the detection of MAGE-A10 mRNA by a multimarker MAGE real-time RT-PCR

primer combination	Mg10_se1 +	Mg10_se2 +
Mg10_anse2	n.d.	37,4
Mg10_anse4	33,7	35,8
Mg10_anse5	38,9	39,9
Mg10_anse6	n.d.	40,8

Table 12: Crossing-Points for the47detection of PBGD and MAGE mRNA by multimarker MAGE real-time RT-PCR in blood and bone marrow aspirates of patients with confirmed relapse of prostate cancer.

CK-ICC = cytokeratin-immunocytochemistry, BM = bone marrow aspirate

patient	CK-ICC	samples	PBGD	MAGE-1	MAGE-2	MAGE-3/6	MAGE-4	MAGE-10	MAGE-12
1.	neg	BM right	26.1	-	-	-	-	-	-
		BM left	26,6	-	-	-	-	-	-
		Blood	22.4	-	-	-	-	-	-
2.	pos	BM right	26.2	-	-	-	-	-	-
		BM left	24.8	-	-	-	-	-	-
		Blood	21.7	-	24.5	-	-	-	-
3.	neg	BM right	23.8	-	-	-	-	-	-
		BM left	23.8	-	-	-	-	-	-
		Blood	-	-	-	-	-	-	-
4.	neg	BM right	21.8	-	24.5	-	-	-	-
		BM left	23.6	-	-	-	-	-	-
		Blood	22.6	-	-	-	-	-	-
5.	neg	BM right	20.8	-	23.9	-	-	-	-
		BM left	24.2	-	-	-	-	-	-
		Blood	23.3	-	-	>36	-	-	-
6.	neg	BM right	23.5	-	-	-	-	-	-
		BM left	21.9	-	-	-	-	-	-
		Blood	21.7	-	-	-	-	-	-
7.	neg	BM right	26.2	-	-	-	-	-	-
		BM left	24.2	-	-	-	-	-	-
		Blood	20.0	-	-	-	-	-	-
8.	neg	BM right	28.2	23.7	11.8	-	33.0	-	-
		BM left	26.9	-	-	-	-	-	-
		Blood	22.7	-	-	-	29.4	-	-
9.	pos	BM right	24.1	-	-	-	-	-	-
		BM left	25.0	-	-	-	-	-	-
		Blood	23.1	-	-	-	-	-	-
10.	pos	BM right	20,9	-	-	-	-	-	-
		BM left	20.2	-	-	-	-	-	-
		Blood	24.9	-	-	-	-	-	-
11.	neg	BM right	22.8	-	-	-	29.2	-	-
		BM left	21.2	-	-	-	-	-	-
		Blood	22.4	-	-	-	-	-	-
12.	neg	BM right	27.3	-	-	-	-	-	-
		BM left	24.7	-	-	-	-	-	-
		Blood	21.6	-	-	-	-	-	-
13.	neg	BM right	-	-	-	-	-	-	-
		BM left	27.0	19.1	-	-	-	-	-
		Blood	23.1	-	-	-	-	-	-
14.	neg	BM right	24.3	13.2	-	-	-	-	-
		BM left	24.1	12.6	23.8	-	-	-	-
		Blood	24.1	-	-	-	-	-	-
15.	neg	BM right	24.1	< 7.0	23.6	-	> 36	-	-
		BM left	24.0	7.2	-	-	-	-	-
		Blood	22.6	-	-	-	-	-	-
16.	neg	BM right	-	-	-	-	-	-	-
		BM left	25.5	18.5	28.8	-	>36	-	-
		Blood	22.1	-	-	-	-	-	-
17.	neg	BM right	23.0	-	-	>36	29.4	-	-
		BM left	29.5	-	-	19.5	-	-	-
		Blood	22.6	-	-	-	-	-	-
18.	neg	BM right	26.1	-	-	19.3	-	-	22.0
		BM left	24.6	-	-	-	-	-	-
		Blood	21.8	21.2	27.5	-	-	-	-
19.	neg	BM right	24.2	-	-	-	16.7	-	-
		BM left	25.0	-	-	-	-	-	-
		Blood	n.a.						
20.	neg	BM right	24.1	-	-	-	-	-	-
		BM left	26.7	-	-	-	-	-	-
		Blood	21.5	-	-	-	-	32.4	-
21.	neg	BM right	23.0	-	-	-	10.6	30.9	9.8
		BM left	30,3	-	-	21.0	< 7.0	-	13.1
		Blood	20.7	-	-	-	-	-	-

Table 13: Crossing-Points for the49detection of PBGD and MAGE mRNA by multimarker MAGE real-time RT-PCR in blood and bone marrow aspirates of patients with low risk for relapse of prostate cancer.

CK-ICC = cytokeratin-immunocytochemistry, BM = bone marrow aspirate

patient	CK-ICC	samples	PBGD	MAGE-1	MAGE-2	MAGE-3/6	MAGE-4	MAGE-10	MAGE-12
1.	pos	BM right	20.0	-	-	-	-	-	-
		BM left	21.6	-	-	-	-	-	-
		Blood	23.0	-	-	-	-	-	-
2.	neg	BM right	20.4	-	-	-	-	-	-
		BM left	19.4	-	-	-	-	-	-
		Blood	20.1	-	-	-	-	-	-
3.	neg	BM right	29.0	-	-	-	-	-	-
		BM left	27.1	-	-	-	-	-	-
		Blood	29.6	-	-	-	-	-	-
4.	neg	BM right	26.5	-	-	-	-	-	-
		BM left	24.7	-	-	-	-	-	-
		Blood	21.2	-	-	-	-	-	-
5.	neg	BM right	25.2	21.4	-	-	-	-	-
		BM left	25.7	21.8	-	-	-	-	-
		Blood	22.3	-	-	-	-	-	-
6.	neg	BM right	26.8	-	-	-	-	-	-
		BM left	27.3	-	-	-	-	-	-
		Blood	23.7	-	-	-	> 36	-	-
7.	pos	BM right	22.0	-	-	-	-	-	-
		BM left	21.7	-	-	-	-	-	-
		Blood	22.9	-	-	-	-	-	-
8.	neg	BM right	23.1	-	-	-	-	-	-
		BM left	22.2	-	-	-	-	-	-
		Blood	22.2	-	-	-	-	-	-
9.	neg	BM right	35.5	-	-	-	30.1	-	-
		BM left	-	-	-	-	-	-	-
		Blood	22.1	-	-	-	-	-	-
10.	neg	BM right	24.1	-	-	-	-	-	-
		BM left	29.6	-	-	-	-	-	-
		Blood	22.8	-	-	-	-	-	-
11.	neg	BM right	23.4	-	-	-	-	-	-
		BM left	22.7	-	-	-	-	-	-
		Blood	22.7	-	-	-	-	-	-
12.	neg	BM right	-	-	-	-	-	-	-
		BM left	24.0	-	-	-	-	-	-
		Blood	23.5	-	-	-	> 36	-	-
13.	neg	BM right	>36	-	-	-	-	-	-
		BM left	25.0	18.9	-	-	20.6	-	-
		Blood	n.a.						
14.	neg	BM right	27.6	23.9	-	-	-	-	-
		BM left	24.8	-	-	-	-	-	-
		Blood	24.4	-	-	-	-	-	-
15.	neg	BM right	30.4	-	-	-	-	-	-
		BM left	26.9	-	-	-	-	-	-
		Blood	21.5	-	-	-	-	-	-
16.	neg	BM right	-	-	-	-	-	-	-
		BM left	23.2	-	-	-	-	-	-
		Blood	21.9	-	-	-	-	-	-
17.	neg	BM right	26.3	14.7	18.5	-	28.2	-	29.6
		BM left	31.2	13.2	-	-	20.8	-	-
		Blood	21.8	-	-	-	> 36	-	-
18.	neg	BM right	24.3	-	-	-	-	-	-
		BM left	24.6	-	-	-	-	-	-
		Blood	21.2	-	-	-	-	-	-